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# Physiological and transcriptional memory in guard cells during repetitive dehydration stress

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## Summary

- Arabidopsis plants subjected to a daily dehydration stress and watered recovery cycle display physiological and transcriptional stress memory. Previously stressed plants have stomatal apertures that remain partially closed during a watered recovery period, facilitating reduced transpiration during a subsequent dehydration stress.

- Guard cells (GCs) display transcriptional memory that is similar to that in leaf tissues for some genes, but display GC-specific transcriptional memory for other genes. The rate-limiting abscisic acid (ABA) biosynthetic genes *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) and *ALDEHYDE OXIDASE 3* (*AAO3*) are expressed at much higher levels in GCs, particularly during the watered recovery interval, relative to their low levels in leaves.

- A genetic analysis using mutants in the ABA signaling pathway indicated that GC stomatal memory is ABA-dependent, and that ABA-dependent *SNF1-RELATED PROTEIN KINASE 2.2* (*SnRK2.2*), *SnRK2.3* and *SnRK2.6* have distinguishable roles in the process. *SnRK2.6* is more important for overall stomatal control, while *SnRK2.2* and *SnRK2.3* are more important for implementing GC stress memory in the subsequent dehydration response.

- Collectively, our results support a model of altered ABA production in GCs that maintains a partially closed stomatal aperture during an overnight watered recovery period.

## Introduction

Plants alter their responses during recurring stresses via an adaptive process termed ‘stress memory’ (Bruce *et al.*, 2007; Byun *et al.*, 2014; To & Kim, 2014). A pre-exposure (priming) to different types of stress may alter subsequent responses by displaying a faster and/or stronger activation of the various response pathways (Liu *et al.*, 2014). This can provide the benefits of enhanced protection against disease or insects (Ton *et al.*, 2005, 2007; Conrath *et al.*, 2006; van Hulten *et al.*, 2006; Jaskiewicz *et al.*, 2011). After a dehydration stress, the extent and speed of the physiological recovery are affected by the severity of the dehydration stress endured (Miyashita *et al.*, 2005; Grzesiak *et al.*, 2006). Despite a fairly rapid recovery in the plant’s water status, a slower rate of photosynthesis and a slow reopening of the stomatal aperture are observed for a period extending up to several days (Galle & Feller, 2007; Galle *et al.*, 2007; Galmes *et al.*, 2007; Flexas *et al.*, 2009; Xu *et al.*, 2009). Arabidopsis plants subjected to a repetitive dehydration stress also display a slower rate of transpiration during a subsequent dehydration stress (Ding *et al.*, 2012). These plants also display genome-wide transcriptional stress memory in leaves as 1963 genes have altered transcript levels during a subsequent dehydration stress relative to their levels in a first dehydration stress (Ding *et al.*, 2013).

Guard cell (GC) regulation of stomatal opening and closing is environmentally responsive to drought, CO<sub>2</sub>, light, humidity,

biotic stresses and different plant hormones (Melotto *et al.*, 2008; Wilkinson & Davies, 2010). Most of the signaling pathways responding to these stimuli include signal transduction via the abscisic acid (ABA) signaling pathway (Joshi-Saha *et al.*, 2011; Lee & Luan, 2012; Nakashima & Yamaguchi-Shinozaki, 2013; Roychoudhury *et al.*, 2013). ABA biosynthesis increases during dehydration stress and both nine-cis-epoxycarotenoid dioxygenase 3 (*NCED3*) and aldehyde oxidase 3 (*AAO3*) appear to catalyze the key rate-limiting steps (Tan *et al.*, 2003; Wan & Li, 2006; Melhorn *et al.*, 2008). ABA biosynthesis appears to be primarily active in vascular tissues and GCs in the aerial parts of the plant (Tan *et al.*, 2003; Koiwai *et al.*, 2004; Ikegami *et al.*, 2009; Bauer *et al.*, 2013).

Once present in a cell, ABA binds to the Pyrabactin Resistance/PYR1-like protein/Regulator component of the ABA receptor (PYR/PYL/RCAR) family of ABA receptor proteins (Ma *et al.*, 2009; Park *et al.*, 2009; Klingler *et al.*, 2010). The ABA-bound receptors bind members of the clade A protein phosphatase 2C (PP2C), which are involved in negatively regulating ABA responses (Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009). The ABA receptor-bound PP2C phosphatases, including ABA Insensitive 1 (*ABI1*), ABA Insensitive 2 (*ABI2*) and Hypersensitive to ABA 1 (*HAB1*), are no longer able to dephosphorylate Snf1 (Sucrose non-fermenting 1)-related protein kinases 2 (*SnRK2s*), allowing the *SnRK2s* to achieve and maintain an active, phosphorylated state to propagate ABA

signaling (Klingler *et al.*, 2010; Yunta *et al.*, 2011; Xie *et al.*, 2012).

The *SnRK2s* are a gene family of serine/threonine protein kinases that mediate ABA-dependent and -independent responses and are organized into three subfamilies: ABA independent group I (*SnRK2.1/2.4/2.5/2.9/2.10*); weakly ABA-activated group II (*SnRK2.7/2.8*), and strongly ABA-activated group III (*SnRK2.2/2.3/2.6*) (Boudsocq *et al.*, 2004; Kulik *et al.*, 2011). The Arabidopsis *SnRK2* ABA-activated group III kinases are expressed in GCs (Fujii *et al.*, 2007) and their critical role in stomatal control was demonstrated via a triple mutation in the group III *snrk2.2/2.3/2.6* kinases that resulted in a loss of stomatal control and severely wilted plants (Fujii & Zhu, 2009; Fujita *et al.*, 2009). *SnRK2.6* (Open stomata 1, OST1) can directly phosphorylate the outward slow anion channel SLAC1 *in vitro*, leading to the activation of the channel to induce stomatal closure (Geiger *et al.*, 2009; Brandt *et al.*, 2012; Lee *et al.*, 2013). *SnRK2.6* also negatively regulates the activity of the K<sup>+</sup> channel (KAT1) K<sup>+</sup> inward ion channel to inhibit its ability to open stomata (Sato *et al.*, 2009). Group III *SnRK2s* also regulate the activity of various transcription factors that bind the ABA-response elements (ABREs) present in many ABA-inducible promoters, such as the ABRE-binding factors (ABFs) (Furihata *et al.*, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Fujii & Zhu, 2009; Fujita *et al.*, 2011).

The occurrence of GC stomatal stress memory, together with the ability to isolate RNA specifically from GCs, provides a system for comparing the cellular response of GC stress memory with GC-specific transcriptional memory responses in a single type of cell (Sirichandra *et al.*, 2009a,b). Here we examine the GC-specific regulation of selected genes to determine their transcriptional memory patterns relative to their behavior in leaf tissues, which are predominantly mesophyll cells. We demonstrate that GCs can display transcriptional memory patterns distinct from those measured in leaves. We also analyzed GC stomatal regulation during repetitive dehydration stress in wild-type plants and in plants with mutations in ABA signaling pathways to gain insight into the genetic control of GC stomatal memory. The results from these experiments emphasize that genes have functionally distinct roles in a first or subsequent dehydration stress, and that ABA-dependent *SnRK2.2* and *SnRK2.3* play a critical role in GC stomatal memory.

## Materials and Methods

### Plant growth and treatments

Wild-type (WT; Columbia-0), *abf2/3/4* (Yoshida *et al.*, 2010), *snrk2.6*, *snrk2.2/2.3*, *snrk2.2/2.3/2.6*, *snrk2.1/2.4/2.5/2.7/2.8/2.9/2.10* (Fujii *et al.*, 2011) and *aba2* (CS6147 allele) mutant *Arabidopsis thaliana* plants were grown in potting soil in growth rooms at 22°C with a 12-h light photoperiod and light intensity of 180  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The day before any treatment, the plants were removed from the soil, any remaining soil from their roots was washed away and their roots were placed in water overnight. The dehydration stress was applied as described previously (Ding

*et al.*, 2012). Briefly, 3-wk-old watered plants (W) were gently blotted onto filter paper to remove water and subjected to an initial air-drying stress for 90 or 120 min (S1). The plants were placed in water for 22 h, corresponding to the recovery period (R1). For a subsequent stress treatment, R1 plants were blotted onto filter paper to remove water and air-dried for 90 or 120 min (S2). One leaf from each of eight to 10 plants was harvested at the different stages, frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation.

### Stomatal aperture and water loss measurements

Stomatal aperture size measurements were performed on 3-wk-old plants by a modification of the glutaraldehyde leaf-fixation methods (Yamaguchi *et al.*, 2007; You *et al.*, 2013). Leaves were fixed in a solution of 4% glutaraldehyde, 10 mM PIPES, pH 7, 5 mM MgCl<sub>2</sub>, and 5 mM EGTA for 6 h at 4°C. The samples were then washed five times in a phosphate-buffered saline (PBS) solution. Forty to sixty stomata from three fixed leaves were examined in each condition using a confocal microscope and cellular autofluorescence (Russo *et al.*, 2010). The autofluorescence of the walls of the leaf epidermal cells and of guard cell extracellular components is highly visible (Supporting Information Fig. S1c; Methods S1). The width of the stomatal pores was measured using IMAGEJ software. The means of the stomatal apertures for three leaves were then used for ANOVA using the statistical software R (R Core Team, 2014) to compare the different conditions for a specific genetic background (Table S1). A comparison of stomatal aperture widths measured by this glutaraldehyde leaf-fixation method to those measured by a method using a rapid ( $\leq 1$  min) confocal microscopic analysis of fresh, unfixed leaves indicated that the two methods provided equivalent results (Fig. S1).

Water loss curves were obtained by weighing the whole plants at different times during the dehydration stress interval. The percent water loss was calculated using the formula: water loss (%) =  $\text{FW}_i / \text{FW}_{0 \text{ min}} \times 100$ , where  $\text{FW}_i$  is the fresh weight for the indicated time, and  $\text{FW}_{0 \text{ min}}$  corresponds to the initial plant weight for the watered or recovery conditions. For all the experiments, two or three replicates, each containing five to eight plants, were used and each experiment was repeated at least three times. The statistical analyses were performed by using an ANOVA, at the  $P=0.05$  level, to compare the significance of the differences between the S1 and S2 curves for each genotype (Table S1).

### Isolation of the guard cells

Approximately 2 g of 3-wk-old plants leaves was excised and blended for 3  $\times$  1 min with a Waring blender in cold water in the presence of actinomycin D (10  $\text{mg l}^{-1}$ ) and cordycepin (50  $\mu\text{M}$ ). Epidermal fragments were collected, washed, and filtered through a 100- $\mu\text{m}$  mesh in three 1.5-ml Eppendorf tubes with cold water in the presence of transcription inhibitors. Blended peels were sonicated to remove contaminating mesophyll and epidermal cells, for 30 s at a power setting of 31%

(Digital Sonifier 450; Branson Ultrasonic Corp., Danbury, CT, USA) for five to six times or until no green chlorophyll contamination was observed. Sonicated tissues were washed after three sonications. A subsequent treatment with RNase A, to remove the RNA which was present from the disrupted epidermal and mesophyll cells, was performed by incubation with 50  $\mu\text{g ml}^{-1}$  RNase A and transcription inhibitors for 10 min at room temperature. The guard cells were washed with cold water in the presence of transcription inhibitors, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further use.

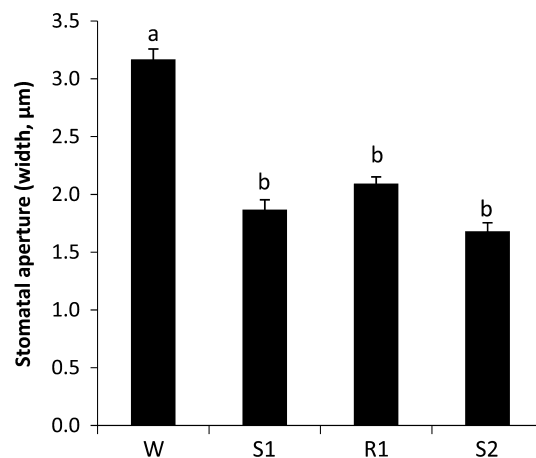
### Reverse transcription and real-time PCR

RNA isolation with TRIzol (Invitrogen, Grand Island, NY, USA), DNase treatment, and reverse transcription were carried out as described previously (Virilouvet *et al.*, 2011). Briefly, the isolated GCs or total leaf tissues were pulverized by bead beating in the presence of TRIzol reagent before the addition of chloroform. Real-time PCR analysis was performed with the cyclerIQ real-time PCR instrument (Bio-Rad, Hercules, CA, USA) and SYBR Green mixture (Bio-Rad). The relative expression of specific genes was quantified with the  $2^{-\Delta\Delta C_t}$  calculation according to the manufacturer's instructions (Bio-Rad (Livak & Schmittgen, 2001)), where  $\Delta\Delta C_t$  is the difference in the threshold cycles of the specific gene and the reference housekeeping gene, which was *actin2* for expression analyses, and then of the specific condition and the well-watered condition. The mean threshold cycle values for the genes of interest were calculated from three technical replicates. The values obtained for the technical replicates (measurements of the same RNA sample) were used for ANOVA analysis with R software to compare the different conditions for a specific tissue or cell type (Table S1). The primers used are given in Table S2.

## Results

### The GC stomatal aperture memory response

Arabidopsis plants subjected to a prior dehydration/recovery cycle show a decreased rate of water loss in a subsequent dehydration stress (Ding *et al.*, 2012). This physiological memory suggests that regulation of the stomatal aperture is occurring. To gain insight into this process, stomatal apertures were measured at the end of the watered (W) period, as well as the end of the first (S1) and second (S2) dehydration stresses, and the end of the 22-h watered recovery period (R1) between S1 and S2 stresses (Fig. 1). We observed that the stomata partially closed during S1, and the extent of the closure was not significantly different from this level at the end of the 22-h R1 watered recovery period or during the subsequent S2 stress (Fig. 1). These results suggest that partially closed stomatal apertures during the watered recovery period are a predominant feature of GC stress memory after dehydration stress, that is, a R1/W GC stomatal stress memory (we will denote the stages being compared in a stress memory analysis). This partially closed state during R1 presumably helps reduce water loss when plants enter the subsequent S2 dehydration stress.



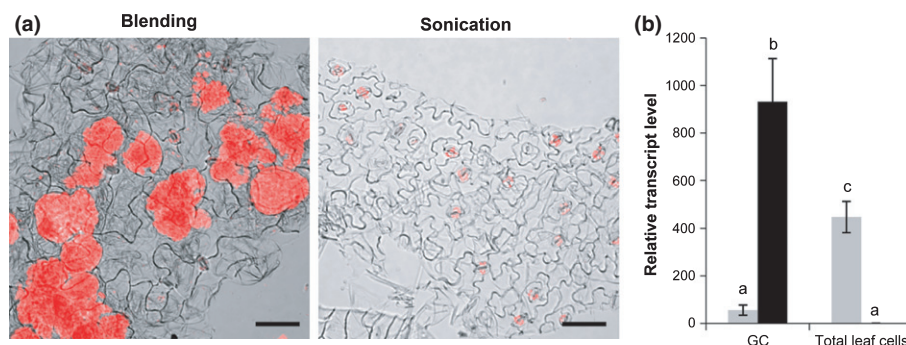
**Fig. 1** Changes in stomatal apertures during repetitive dehydration stress. Stomatal apertures were measured in 3-wk-old Arabidopsis Columbia (Col-0) plants after a watered period (W), a first (S1) or second (S2) stress of 90 min of air-drying, or a 22-h watered recovery period (R1). The values correspond to the mean width of 18 stomata for three individual plants ( $n = 3$ ) for each condition, with the SE of the mean shown. Different letters indicate that the difference is significant ( $P < 0.05$ ).

### Isolation of GC-specific RNAs

We next sought to examine selected gene transcript levels in GCs. We developed a nonprotoplast method that rapidly isolates intact GCs while removing and/or disrupting the other leaf cells. This GC RNA isolation method combines two steps from previously published protocols. First, a Waring blender is used to chop leaves into small epidermal fragments (Pandey *et al.*, 2002). After filtering out larger leaf fragments, the remaining intact tissue fragments are predominantly epidermal peels containing some intact epidermal cells and intact GCs (Fig. 2a). These epidermal peels are sonicated at a power level sufficient to disrupt the cell wall of epidermal cells but not the stronger cell wall of GCs (Cornish & Zeevaart, 1986). Microscopic examination for the presence of chloroplasts serves as an indication of cell integrity and showed that only GCs were intact after this sonication procedure (Fig. 2a). Both the blending and sonication steps are conducted at  $4^{\circ}\text{C}$  and in the presence of transcription inhibitors (Leonhardt *et al.*, 2004) to minimize the production of new transcripts during these steps. The intact GC preparations are treated with RNase A to digest any RNA released from the disrupted cells. The GCs are then pulverized by bead beating in the presence of TRIzol for RNA isolation (see the Materials and Methods section).

The cellular specificity of the RNA isolated from the GC preparations was then estimated by measuring the levels of GC1, a GC-specific transcript (Yang *et al.*, 2008), and  $\beta$ -carbonic anhydrase 1 ( $\beta\text{CA1}$ ), which is expressed in GCs and mesophyll cells, but is *c.* 4-fold more abundant in mesophyll cells (Hu *et al.*, 2010). As expected, the RNA isolated from GCs had a high ratio of GC1 compared with  $\beta\text{CA1}$  (Fig. 2b). By contrast, in RNA isolated from total leaf tissue,  $\beta\text{CA1}$  RNA levels were higher, and GC1 was 931-fold less abundant than in GCs (Fig. 2b). These results indicate that this blender/sonication protocol rapidly isolates RNAs that are predominantly from GCs.





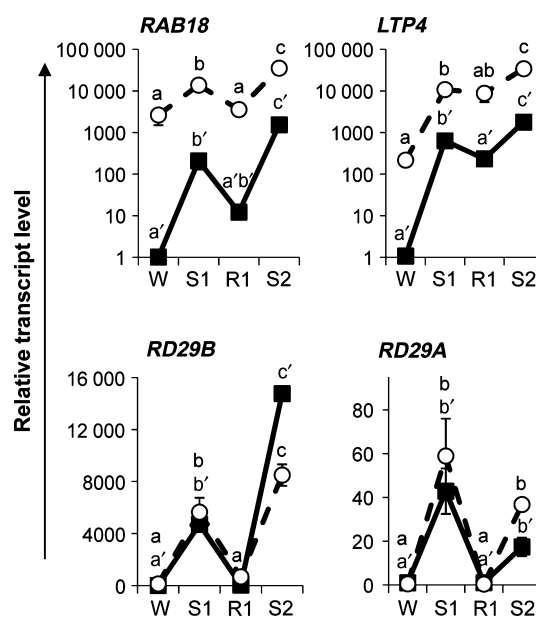
**Fig. 2** Guard cell isolation and transcript analysis. (a) Images ( $\times 20$ ) of the chloroplast fluorescence (red) in leaf epidermal peels immediately after blending (left panel) and sonication (right panel) during the guard cell isolation procedure from 3-wk-old *Arabidopsis Columbia* (Col-0) leaves. Bars, 50  $\mu\text{m}$ . (b) Relative levels of guard cell (GC)-specific protein 1 (*GC1*) (gray bars) as well as  $\beta$ -CARBONIC ANHYDRASE 1 (*βCA1*) (black bars) transcripts that are expressed in GCs and mesophyll cells. Real-time quantitative PCR was performed on RNA isolated from GCs and total leaf tissue from 3-wk-old seedlings. Bars correspond to the values of a representative experiment; mean  $\pm$  SEM of three technical replicates. The experiment was performed three times. Different letters indicate that the difference is significant ( $P < 0.05$ ).

### The transcriptional memory response in GCs

We next evaluated transcriptional memory responses of specific genes in GCs using the above RNA isolation protocol and compared these responses with those obtained in leaf tissues. We first analyzed four genes, *ras-related small GTPase homologue B18* (*RAB18*), *lipid transfer protein 4* (*LTP4*), *responsive to desiccation 29A* (*RD29A*) and *responsive to desiccation 29B* (*RD29B*), that are known to be expressed and/or induced by ABA or dehydration stress in GCs (Leonhardt *et al.*, 2004; Wang *et al.*, 2011). Importantly, we previously found that *RAB18*, *LTP4*, and *RD29B* displayed S3/S1 transcriptional memory in leaf tissues as they were induced to higher levels in a third S3 dehydration stress relative to a first S1 dehydration stress (Ding *et al.*, 2012). By contrast, *RD29A* did not display S3/S1 transcriptional memory in leaf tissues as it was induced to about the same or lower levels during repetitive stress cycles (Ding *et al.*, 2012). The results of our analysis of *RAB18*, *LTP4*, *RD29A* and *RD29B* transcript profiles in whole leaves were in agreement with our prior publication (Ding *et al.*, 2012), as *RAB18*, *LTP4* and *RD29B* displayed significant S2/S1 transcriptional memory as they were induced at higher levels during S2 relative to S1, while *RD29A* was not (Fig. 3). In GCs, these genes generally had transcriptional response patterns that were similar to their memory response in leaf tissues (Fig. 3). The higher basal levels of *RAB18* and *LTP4* in the W state appear to be a GC-specific feature of these genes that is consistent with previous reports of their transcript levels (Leonhardt *et al.*, 2004; Wang *et al.*, 2011). These results demonstrate that S2/S1 transcriptional memory occurs in GCs as well as mesophyll cells, and suggest that the dehydration stress transcriptional responses are added to the high basal levels of expression observed for *RAB18* and *LTP4* in GCs.

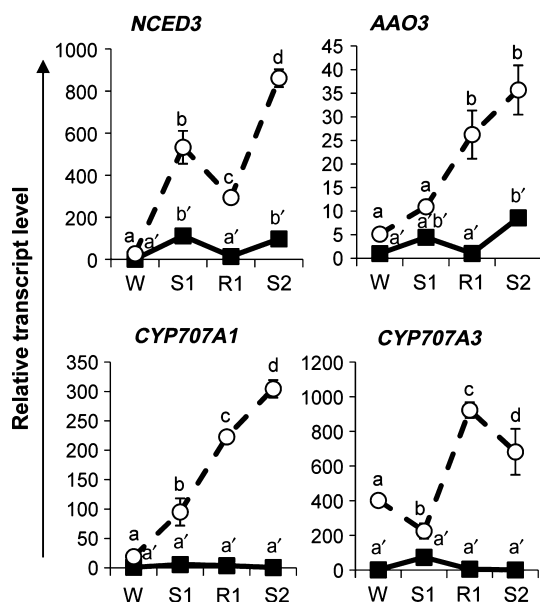
### ABA biosynthesis and degradation gene transcripts in GCs

A puzzling observation was that stomatal apertures remained partially closed at the end of the 22-h R1 interval (Fig. 1), suggesting that elevated ABA concentrations were occurring during R1. However, the ABA-responsive *RD29A* and *RD29B*



**Fig. 3** Transcript levels of *RAB18*, *LTP4*, *RD29A*, and *RD29B* in guard cells (GCs) and total leaves. Transcript levels were measured in 3-wk-old *Arabidopsis Columbia* (Col-0) plants using real-time quantitative PCR on RNA isolated from GCs (open circles) or total leaf cells (closed squares) in watered (W), first stress (S1), recovery (R1) and second stress (S2) conditions. Bars correspond to the values of a representative experiment; mean  $\pm$  SEM of three technical replicates. The experiment was repeated at least three times. Different letters within the a–c (GC) or a'–c' (leaf cells) series indicate that the difference is significant ( $P < 0.05$ ). Note that the scale for *RAB18* and *LTP4* is  $\log_{10}$ .

genes returned to basal levels in GCs at the end of the 22-h R1 interval, indicating that low levels of ABA signaling were being perceived. To help resolve this contradiction, we measured transcript levels of two genes involved in ABA biosynthesis, *NCED3* and *AAO3* (Iuchi *et al.*, 2001; Tan *et al.*, 2003; Wan & Li, 2006; Melhorn *et al.*, 2008), as well as two genes involved in ABA degradation, cytochrome P450, family 707, subfamily A, polypeptide 1 and 3: *CYP707A1* and *CYP707A3* (Kushiro *et al.*, 2004).



**Fig. 4** Transcript levels of abscisic acid (ABA) metabolism genes in the guard cells (GCs) and total leaves. The transcript levels were measured in 3-wk-old *Arabidopsis* Columbia (Col-0) plants using real-time quantitative PCR on RNA from isolated GCs (open circles) or total leaf cells (closed squares) in watered (W), first stress (S1), recovery (R1) and second stress (S2) conditions. Bars correspond to the values of a representative experiment; mean  $\pm$  SEM of two technical replicates. Each experiment was repeated at least three times. Different letters within the a–d (GC) or a'–b' (leaf) series indicate that the difference is significant ( $P < 0.05$ ).

Transcript levels of these four genes were measured in GC and leaf RNA (Fig. 4). In leaves, *NCED3* was induced to similar levels during S1 and S2 dehydration stresses and restored to basal watered levels at the end of the watered R1 recovery (Fig. 4). By contrast, in GCs *NCED3* was induced to much higher levels in S1, remained at fairly high levels at the end R1, and then was induced to significantly higher levels in S2 than in S1 (Fig. 4). Therefore, *NCED3* displayed an S2/S1 and R1/W transcriptional memory pattern in GCs that was different from its response in leaves. The transcript level of *AAO3* was higher in R1 than in S1 in GCs, while transcript levels were back to watered levels in R1 in leaves (Fig. 4). Therefore, *AAO3* also displayed GC-specific S2/S1 and R1/W transcriptional memory patterns.

A simple interpretation of the elevated *NCED3* and *AAO3* transcript levels in GCs during R1 is that the rate of ABA biosynthesis was higher during R1 than in the W condition. However, this presumed increase in the rate of ABA biosynthesis was accompanied by an increase in GCs in the transcript levels of *CYP707A1* and *CYP707A3*, which encode ABA degradation enzymes (Fig. 4). Both *CYP707A1* and *CYP707A3* transcripts showed S2/S1 and R1/W GC-specific transcriptional memory responses that were distinct from their much lower transcript levels found in total leaf cells (Fig. 4). In summary, all four ABA metabolism genes displayed GC-specific S2/S1 and R1/W transcriptional memory patterns that were different from those observed in leaves, suggesting that increased ABA biosynthesis and degradation occurred concurrently during the R1 watered recovery period.

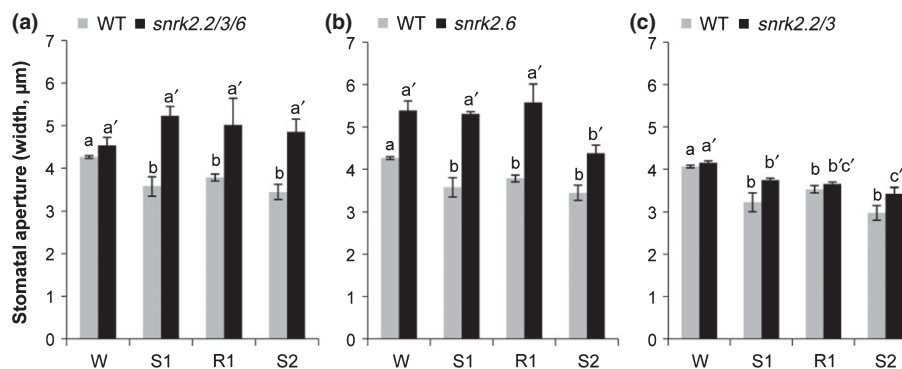
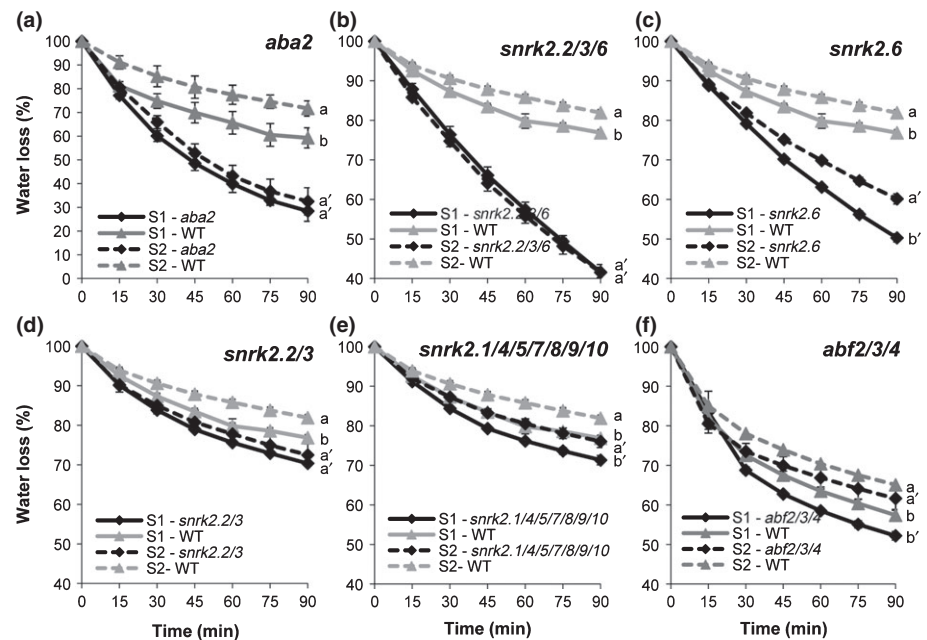
## The ABA signaling pathway regulates the GC stomatal memory response

The size of stomatal apertures in WT plants (Fig. 1) affects the rate of water loss from plants during repetitive dehydration stress. Measurement of the rate of water loss from plants experiencing their first dehydration stress (S1) or second dehydration stress (S2) demonstrates this difference (e.g. see the S1 and S2 water loss curves for WT plants in Fig. 5a). This difference in the rates of water loss provides a convenient assay for measuring S2/S1 GC stomatal memory in WT and mutant plants. We refer to this assay as a S2/S1 GC stomatal memory assay because it measures water loss during the S1 and S2 dehydration stresses. Note that the rate of water loss during S2 is affected by the R1 stomatal aperture being smaller at the beginning of S2 than the W stomatal aperture size at the beginning of S1 (Fig. 1). Thus, the S2/S1 GC stomatal memory assay is affected by R1 changes.

To gain further insight into the role of ABA in S2/S1 GC stomatal memory, the rate of water loss of various mutants involved in ABA-mediated stomatal responses was investigated during S1 and S2 dehydration stress. We found that the *aba2* mutant, which is deficient in ABA biosynthesis (Nambara *et al.*, 1998), had rapid rates of water loss that were not significantly different in S1 and S2 (Fig. 5a). This suggests that very little S2/S1 GC stomatal memory can occur at the very low or absent levels of ABA in *aba2* plants. This ABA signaling for S2/S1 GC stomatal memory was mediated predominantly by SnRK2.2, SnRK2.3 and SnRK2.6, as a *snrk2.2/2.3/2.6* triple mutant had rapid rates of water loss that were not significantly different in S1 and S2 (Fig. 5b). The *snrk2.6* mutant displayed a rapid rate of water loss in S1, but had a significantly slower rate during S2, demonstrating that *snrk2.6* retains S2/S1 GC stomatal memory (Fig. 5c). This result contrasts with the result for the *snrk2.2/2.3* double mutant, which had rates of water loss in S1 and S2 that were not significantly different (Fig. 5d). A *snrk2.1/2.4/2.5/2.7/2.8/2.9/2.10* septuple mutant displayed S2/S1 GC stomatal memory, as the slower rate of water loss in S2 was significantly different from the faster rate in S1 (Fig. 5e).

The correlation of the rate of water loss with stomatal apertures was analyzed by measuring stomatal apertures of *snrk2.6*, *snrk2.2/2.3*, and *snrk2.2/2.3/2.6* mutants during repetitive dehydration stress (Fig. 6). The *snrk2.2/2.3/2.6* triple mutant stomatal apertures did not change under repetitive dehydration stress (Fig. 6a), in agreement with its similar rates of water loss in S1 and S2 (Fig. 5b). Stomatal apertures of *snrk2.6* were similar in the watered W, initial S1, and R1 recovery conditions, indicating very little response to these conditions. However, *snrk2.6* stomata were significantly smaller during S2 conditions (Fig. 6b), in agreement with the slower rate of water loss in S2 (Fig. 5c). Stomatal apertures of the *snrk2.2/2.3* mutant displayed R1/W GC stomatal memory, as R1 apertures were significantly smaller than W apertures (Fig. 6c). Most importantly, a comparison of the lack of stomatal control in the *snrk2.2/2.3/2.6* mutant (Fig. 6a) with the partial stomatal closure during S2 for the *snrk2.6* mutant (Fig. 6b) revealed that SnRK2.2 and SnRK2.3 are particularly important for GC stomatal closure during S2.

**Fig. 5** The involvement of abscisic acid (ABA) signaling in guard cell (GC) stomatal memory. Three-week-old (a) *aba2*, (b) *Sucrose non-fermenting 1 (snf1)*-related protein kinase 2.2 (*snrk2.2*)/3/6, (c) *snrk2.6*, (d) *snrk2.2/3*, (e) *snrk2.1/4/5/7/8/9/10* and (f) ABA-response element binding factor 2 (*abf2*)/3/4 mutant and wild-type Arabidopsis Columbia (Col-0) (WT) plants were analyzed for water loss during a first (S1) and second (S2) 90 min of air-drying stress. Error bars are SE of the mean of two or three values of at least three plants. The experiment was repeated at least three times. Different letters within the a–b (WT) or a'–b' (mutant) series indicate that the difference is significant ( $P < 0.05$ ).



**Fig. 6** Effect of mutations on stomatal aperture during repetitive dehydration stress. Stomatal apertures of 3-wk-old Arabidopsis wild-type Columbia (Col-0) (WT) and (a) *sucrose non-fermenting 1 (snf1)*-related protein kinase 2.2 (*snrk2.2*)/2.3/2.6, (b) *snrk2.6*, and (c) *snrk2.2/2.3* mutant plants were measured by microscopy after fixation of the stomata. Plants were harvested after a watered period (W), a first (S1) or second (S2) 90 min of air-drying stress, or a 22-h recovery period (R1). The values correspond to the mean width of 20–60 stomata for three individual plants ( $n = 3$ ) for each condition, with standard error bars. Different letters within the a–b (WT) or a'–c' (mutant) series indicate that the difference is significant ( $P < 0.05$ ).

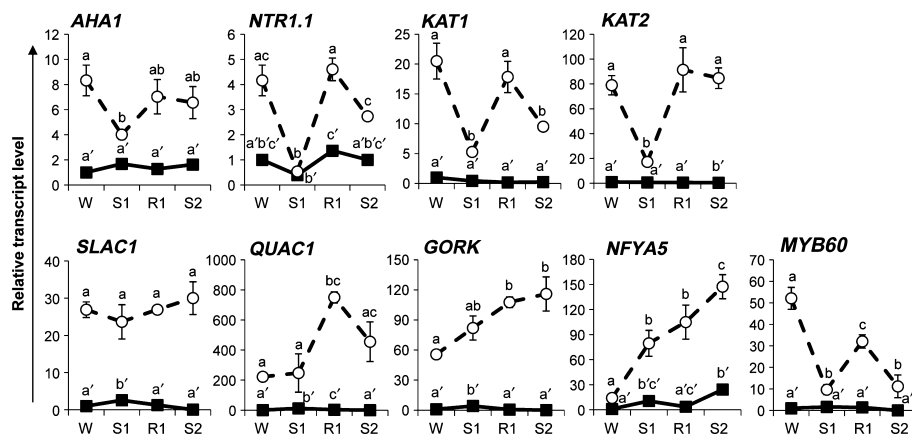
ABF2, ABF3 and ABF4 are SnRK2-regulated transcription factors that activate many ABA-responsive genes (Fujita *et al.*, 2005; Yoshida *et al.*, 2010). We found that the *abf2/3/4* triple mutant had a higher rate of water loss in S1 and S2 dehydration stresses, relative to the WT control plants (Fig. 5f). However, *abf2/3/4* plants lost water at a significantly slower rate in S2 than in S1 (Fig. 5f). These results demonstrate that ABF2, ABF3 and ABF4 can affect the degree of stomatal closure during dehydration stress, in agreement with earlier reports (Yoshida *et al.*, 2010), and that *abf2/3/4* mutant plants retain S2/S1 GC stomatal memory.

#### Transcriptional memory of membrane proteins and transcription factors in GCs

We next measured transcript levels of seven key ion channel and transporter proteins (reviewed in Kim *et al.*, 2010), namely

H<sup>+</sup>ATPase 1 (AHA1), nitrate transporter 1 (NRT1.1), potassium channel in *Arabidopsis thaliana* 1 (KAT1), potassium channel in *Arabidopsis thaliana* 2 (KAT2), slow anion channel associated 1 (SLAC1), quick anion channel associated 1 (QUAC1) and gated outwardly-rectifying K<sup>+</sup> channel (GORK), to gain further insight into transcriptional memory in GCs. AHA1 is a member of the H<sup>+</sup>-ATPase family of P-type ATPases, which are all expressed in the GCs and mediate the efflux of H<sup>+</sup> from the GCs during the opening of the stomata (Ueno *et al.*, 2005). NRT1.1, a nitrate transporter, also functions in stomatal opening (Guo *et al.*, 2003). KAT1 and KAT2 encode potassium channels which mediate K<sup>+</sup> uptake during stomatal opening (Schachtman *et al.*, 1992; Pilot *et al.*, 2001), whereas GORK encodes the K<sup>+</sup><sub>out</sub> channel involved in stomatal closure (Hosy *et al.*, 2003). SLAC1 and QUAC1 encode slow (S-type) and rapid (R-type) ion channels, respectively, and mediate anion efflux during the closure of the





**Fig. 7** Transcript levels of genes involved in stomatal regulation. Transcript levels were measured in 3-wk-old *Arabidopsis Columbia* (Col-0) plants using real-time quantitative PCR on RNA from isolated guard cells (GCs; open circles) or total leaf cells (closed squares) after a watered (W), first stress (S1), 22-h recovery (R1), or second stress (S2) treatment. The values of a representative experiment are shown; mean  $\pm$  SEM of three technical replicates. The experiment was repeated at least three times. Different letters within the a–c (GC) or a'–c' (leaf) series indicate that the difference is significant ( $P < 0.05$ ).

stomata (Mori *et al.*, 2006; Vahisalu *et al.*, 2008; Dreyer *et al.*, 2012; Imes *et al.*, 2013).

These seven genes were all expressed at higher levels in GCs than in leaves (Fig. 7). The four genes involved in stomatal opening, *AHA1*, *NTR1.1*, *KAT1* and *KAT2*, were all expressed at significantly lower levels during S1 relative to their W state, consistent with closing of the stomatal aperture during S1. Their transcript levels during R1 were not significantly different from their levels during the W state. However, *NTR1.1* and *KAT2* displayed S2/S1 transcriptional memory as their S1 and S2 transcript levels were significantly different, while those of *AHA1* and *KAT1* were not (Fig. 7). Note that, while stomatal apertures remained partially closed in R1 (Fig. 1), the GC transcript levels of these four genes returned to levels not significantly different from their levels in the W state (Fig. 7).

The three genes involved in stomatal closure, *SLAC1*, *QUAC1*, and *GORK*, had different responses (Fig. 7). *SLAC1* transcript levels did not significantly change in GCs during dehydration stress. *QUAC1* transcripts were higher in R1 in GCs relative to the W control, but were not significantly different in S1 and S2. This R1/W transcriptional memory displayed by *QUAC1* was similar in GCs and leaf cells (Fig. 7). *GORK* transcript levels were significantly higher in R1 relative to W levels, thereby displaying R1/W transcriptional memory in GCs but not in leaf cells (Fig. 7). The R1/W transcriptional memory that resulted in higher levels of *QUAC1* and *GORK* transcripts in R1 suggests a potential involvement of the R-type ion and  $K^+$ <sub>out</sub> channels, respectively, in the partial closure of the stomatal aperture during the recovery period.

We also measured the transcript levels of two transcription factors, namely nuclear factor Y subunit A5 (NFYA5) and MYB60, both of which are involved in regulating GC apertures. Nuclear factor Y (NFY) is a ubiquitous transcription factor composed of three distinct subunits (NFYA, NFYB and NFYC). The *Arabidopsis* *NFYA5* transcript is strongly induced by drought stress in an ABA-dependent manner (Li *et al.*, 2008). Functionally, *NFYA5* appears to facilitate stomatal closing as *nfya5* mutants lose water at a faster rate, while plants overexpressing *NFYA5* lose water at a slower rate (Li *et al.*, 2008). In leaves, *NFYA5* transcript levels increased in S1, decreased in R1 and then increased to levels in S2 similar to those in S1 (Fig. 7). However, in GCs,

*NFYA5* was expressed at increasingly higher levels in S1, R1 and S2, thus displaying S2/S1 and R1/W transcriptional memory in GCs (Fig. 7). The generally higher *NFYA5* transcript levels in GCs in S1, R1 and S2, together with its apparent functional role in stomatal closure, suggest that *NFYA5* is facilitating the closure of stomata that is occurring during S1, R1 and S2.

MYB60 is a transcription factor that functions in stomatal opening and which is down-regulated in response to ABA (Cominelli *et al.*, 2005), which presumably facilitates stomatal closing. We found that its transcript levels were fairly low and unchanging in leaves but it was expressed at high levels in GCs in the W condition (Fig. 7), in agreement with *MYB60* GC-specific promoter expression (Rusconi *et al.*, 2013). In GCs, *MYB60* expression was significantly lower in S1, R1 and S2 compared with its transcript levels in the W state. Its negative R1/W transcriptional memory, which means transcript levels were lower in the R1 recovery state than the W state, was consistent with attenuation of the role of MYB60 in stomatal opening during the watered R1 recovery interval when the stomatal aperture was partially closed.

## Discussion

### Physiological stress memory response improves tolerance to subsequent dehydration stress

Previous studies have observed that stomatal re-opening is incomplete during a watered recovery after drought stress conditions (Davies & Kozlowski, 1977; Ni & Pallardy, 1992; Loewenstein & Pallardy, 2002). Whereas leaf water potential had largely recovered within 6 h of rewatering in five species, *Quercus alba*, *Quercus velutina* (black oak), *Juglans nigra*, black willow (*Salix nigra*) and eastern cottonwood (*Populus deltoides*), stomatal conductance did not recover to control levels for at least one full day (Loewenstein & Pallardy, 2002). Our results indicate that *Arabidopsis* stomatal apertures also do not open to levels typical for watered conditions for at least 1 d. In our repetitive dehydration stress system, wherein dehydration stress was imposed on a 24-h cycle, the partially closed stomata at the end of the 22-h R1 recovery interval facilitated the slower rates of water loss. We propose that this is analogous to what happens during a diurnal cycle during drought stress. Plants experiencing



dehydration stress during the day often recover turgor pressure at night, thereby temporarily alleviating dehydration stress conditions (Tardieu *et al.*, 2010). The plant's ability to use physiological and transcriptional stress memory to maintain its preparation for dehydration stress presumably improves its tolerance and reduces its water loss during the next day's stress. Upon sustained water availability for several days, these memory processes are lost, allowing the plant to return to its nonstressed growth patterns (Ding *et al.*, 2012).

### ABA concentrations are likely to be partially elevated in GCs during recovery

Our results demonstrate that GC stomatal memory requires ABA, as the rates of water loss in S1 and S2 dehydration stresses were approximately the same in an *aba2* mutant background. Elevated residual ABA concentrations and/or increased sensitivity to ABA have been proposed as potential mechanisms by which GC stomatal memory persists during watered conditions after a dehydration stress. For example, ABA concentrations in the xylem sap of recovering plants are two to three times above control concentrations 1 d after watering, although ABA returns to control concentrations before complete stomatal opening (Loewenstein & Pallardy, 2002). ABA concentrations in Arabidopsis plants subjected to our repetitive stress cycles have residual ABA concentrations two to three times above control concentrations during recovery periods of up to several days (Ding *et al.*, 2012). ABA concentrations in GCs in dehydration-stressed *Vicia faba* leaves persist at elevated levels for 4–6 h, but not 8 h, in this detached leaf system (Harris *et al.*, 1988; Harris & Outlaw, 1991). The higher transcript levels of the two key regulatory genes of the ABA biosynthetic pathway, *NCED3* and *AAO3*, suggest that biosynthesis of ABA is active during the watered recovery in GCs. However, as we also observed an increase in the transcript levels of the ABA degradation genes *CYP707A1* and *CYP707A3*, the net effect on ABA concentrations is unclear. Different subcellular sites of ABA biosynthesis, degradation, conjugation, export, and import may also be able to affect localized subcellular concentrations of ABA. In particular, different subcellular ABA concentrations could trigger plasma membrane stomatal responses and nuclear transcriptional responses differentially at low cellular ABA levels. (Nambara & Marion-Poll, 2005; Xu *et al.*, 2013). As measuring ABA concentrations specifically in GCs is extremely difficult (Harris *et al.*, 1988; Harris & Outlaw, 1991), we used an ABA-responsive gene in GCs as an indirect indicator of ABA concentrations.

The endogenous *RD29B* gene is highly responsive to ABA concentrations via activation of ABF2/3/4 by SnRK2.2/2.3/2.6 phosphorylation (Kang *et al.*, 2002; Fujita *et al.*, 2005, 2009; Fujii *et al.*, 2009; Yoshida *et al.*, 2010). The observation that *RD29B* was not induced at the end of an R1 watered recovery period indicates that it was not receiving an ABA signal. This result is puzzling as ABA signaling proceeds via a common SnRK2.2/2.3/2.6 step for both stomatal regulation and *RD29B* induction (Fujii & Zhu, 2009; Fujita *et al.*, 2011) and both these processes are likely to have fully responded to ABA

concentrations by the end of the 22-h recovery interval. A possible explanation is that the stomatal regulatory pathway responds at lower concentrations of ABA than the transcriptional regulatory pathway. Hypothetically, this could be a result of a higher affinity of SnRK2.2/2.3/2.6 for their substrates in the stomatal regulatory pathway and/or a slower rate of dephosphorylation of these substrates. Presumably, higher concentrations of ABA and SnRK2.2/2.3/2.6 activity are required to activate the ABF2/3/4 pathway that regulates *RD29B* (Fujita *et al.*, 2009; Yoshida *et al.*, 2010). Therefore, our data do not support the hypothesis that an overall increased sensitivity to ABA is occurring at the SnRK2.2/2.3/2.6 activation step that is shared in the stomatal and transcriptional ABA-response pathways.

A comparison of GC-specific and leaf expression analyses may resolve the puzzle as to why stomata continue to stay partially closed after ABA concentrations return to control values in leaves or xylem sap in many plants (Loewenstein & Pallardy, 2002). Total leaf *NCED3* and *AAO3* transcript levels return to control levels during the watered recovery period, while GC-specific transcripts of these genes remain elevated. This suggests that ABA measurements at the whole leaf level or in xylem sap are unlikely to detect ABA being specifically produced only in GCs during the watered recovery interval. Our results suggest that low concentrations of residual ABA and/or ABA newly synthesized in GCs are preferentially activating the stomatal control pathway at concentrations sufficient for partial closure of the stomata, but not at concentrations sufficient to activate the ABF2/3/4 transcriptional pathway in GCs during the watered recovery interval.

### Some genes have different transcriptional memory patterns in GCs and mesophyll cells

Transcriptional memory in GCs displayed a variety of patterns relative to the behavior of the same genes in leaves, which are comprised predominantly of mesophyll cells. The *RAB18*, *LTP4*, *RD29A* and *RD29B* structural genes had elevated levels of transcripts in S1 and higher levels in S2 in GCs and leaves, thereby displaying an S2/S1 transcriptional memory response in agreement with their previously reported S3/S1 transcriptional memory patterns in leaves (Ding *et al.*, 2012). The main difference in GCs was that the basal levels of *RAB18* and *LTP4* were much higher in nonstressed GCs than in leaves, in agreement with prior studies of GCs (Wang *et al.*, 2011).

By contrast, 10 genes with regulatory roles in GCs generally showed GC-specific patterns of transcriptional memory that were often distinct from their behavior in leaves. These included the ABA biosynthesis (*NCED3* and *AAO3*) or degrading (*CYP707A1* and *CYP707A3*) genes, which had higher levels of expression in GCs and displayed R1/W transcriptional memory in GCs but not in leaf cells. The ion channels, ion transporters, and membrane protein genes analyzed had transcript profiles in GCs that were also generally different from their leaf profiles. *AHA1*, *NRT1.1* (*CHL1*), *KAT1*, and *KAT2* are involved in stomatal opening and displayed decreased transcript levels in S1 in GCs, a result consistent with decreased stomatal opening during S1.

However, during the R1 recovery interval their transcript levels were restored to approximately their levels in W conditions, despite maintaining a partially closed stomatal aperture. Similarly, three genes involved in stomatal closure, *SLAC1*, *QUAC1* and *GORK*, were expressed at higher levels in GCs than leaf cells, but differed in their GC stress responses. *SLAC1* transcript levels did not change during dehydration stress, while *QUAC1* and *GORK* displayed R1/W transcriptional memory in GCs, presumably facilitating partial stomatal closure during the R1 recovery period. These gene products are additionally regulated by post-translational mechanisms such as phosphorylation/dephosphorylation (Ho *et al.*, 2009; Sato *et al.*, 2009; Lebaudy *et al.*, 2010; Caesar *et al.*, 2011).

The NFYA5 and MYB60 transcription factors have opposite functional roles in stomatal regulation (Cominelli *et al.*, 2005; Li *et al.*, 2008) and displayed different GC-specific transcriptional memory patterns. *NFYA5* displayed a positive R1/W transcriptional memory while *MYB60* displayed a negative R1/W transcriptional memory as its transcript levels were lower in R1 than in the W state. The directional increase in *NFYA5* and decrease in *MYB60* transcript levels in GCs in R1 are consistent with the partial stomatal closure during R1 and their functional roles in the induction of stomatal closure and stomatal opening, respectively.

### SnRK2.2, SnRK2.3, and SnRK2.6 have functionally distinguishable roles in S2/S1 GC stomatal stress responses

The group III ABA-dependent SnRK2.2, SnRK2.3, and SnRK2.6 play important roles in stomatal regulation. SnRK2.6 phosphorylates ion channels, ion transporters, and other membrane proteins (Vahisalu *et al.*, 2008; Sato *et al.*, 2009; Sirichandra *et al.*, 2009a,b). SnRK2.2 and SnRK2.3 have less well-defined roles but are known to activate *SLAC1* (Geiger *et al.*, 2009). Our results indicate that SnRK2.2 and SnRK2.3 are more important for regulation of stomatal apertures during S2 than during S1. This conclusion is based on several observations in the different *snrk2* mutant backgrounds. First, the *snrk2.2/2.3/2.6* triple mutants rapidly lose water at equal rates in S1 and S2, and essentially display little control of stomatal apertures (Fujii & Zhu, 2009). This result indicates that S2/S1 GC stomatal memory control is lost in this triple mutant. Secondly, the *snrk2.6* mutant displays very little stomatal closing during S1 but does in S2, implicating SnRK2.2 and SnRK2.3 in S2 stomatal closure. Thirdly, *snrk2.2/snrk2.3* plants display similar rates of water loss in S1 and S2. Thereby, SnRK2.2 and SnRK2.3 are identified as key regulators of S2/S1 GC stomatal stress memory, regulating stomatal apertures differentially in S2. These stomatal stress memory effects are not mediated by ABF2, ABF3, and ABF4 controlled transcriptional responses as the *abf2/3/4* triple mutant still displays S2/S1 GC stomatal stress memory.

Taken together, our results demonstrate that GCs display S2/S1 and/or R1/W transcriptional stress memory that is often distinct from that in leaves and elucidate some of the mechanisms facilitating S2/S1 GC stomatal stress memory, which probably

includes contributions from the R1 recovery period as well. These results support the hypothesis that ABA concentrations are partially elevated in GCs during watered recovery intervals via GC-specific biosynthesis of ABA, at concentrations sufficient for partial stomatal closure but not sufficient for inducing ABA-dependent transcriptional responses. This differential response to low concentrations of ABA might be enhanced by the subcellular specificities of ABA biosynthesis, degradation, conjugation, and compartmentalization (Nambara & Marion-Poll, 2005; Xu *et al.*, 2013). We propose that the ABA-dependent mechanism of partial stomatal closure during watered recovery intervals facilitates plant adaptation to the diurnal cycle of dehydration stress that occurs during periods of low water potential such as drought stress conditions.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Comparison of stomatal apertures measured by microscopic analysis of fresh leaves or chemically fixed leaves and mea-

surement of stomatal apertures in fresh unfixed leaves by guard cell autofluorescence visualized by confocal laser scanning microscopy.

**Table S1** ANOVA for the stomatal aperture measurements, the transcript levels in guard cells and total leaf cells and the water loss measurements

**Table S2** Primers used for the real-time PCR

**Method S1** Observation of the stomatal aperture of the leaves by confocal microscopy.

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